

Table 1. Effect of increasing concentrations of lipopolysaccharides (LPS) with (+) or without (–) anti-bovine tumor necrosis factor (TNF)- α mAb (A) or of TNF- α (B) on the expression of CD11b (MFI) in circulating polymorphonuclear leukocytes (PMN). Data represent means \pm standard error (s_x). No differences could be found between early- (EL), peak- (PL), and midlactation (ML). Statistically significant differences between control and samples stimulated with LPS (I) or TNF- α (II), and between control and samples preincubated with anti-TNF- α mAb (III) are indicated

A.

LPS (ng/mL)	anti-TNF- α mAb	EL ($n = 8$)	PL ($n = 8$)	ML ($n = 8$)	Average ($n = 24$)	I. Control vs. LPS	III. mAb effect
0	—	28.14 \pm 1.23	31.44 \pm 2.08	32.03 \pm 3.11	30.54 \pm 1.26	—	—
0	+	28.69 \pm 1.25	31.92 \pm 2.02	31.34 \pm 2.88	30.65 \pm 1.17	—	ns
1	—	32.70 \pm 1.86	36.60 \pm 2.81	39.20 \pm 4.30	36.16 \pm 1.88	$P < 0.001$	—
1	+	29.76 \pm 1.30	35.12 \pm 2.40	34.18 \pm 3.63	33.02 \pm 1.51	—	$P < 0.001$
10 ²	—	32.63 \pm 1.51	38.42 \pm 2.95	40.12 \pm 4.74	37.06 \pm 1.90	$P < 0.001$	—
10 ²	+	31.39 \pm 1.65	37.00 \pm 2.78	36.76 \pm 4.03	35.05 \pm 1.66	—	$P < 0.001$
10 ⁴	—	32.09 \pm 1.85	37.42 \pm 2.61	37.63 \pm 4.42	35.71 \pm 1.81	$P < 0.001$	—
10 ⁴	+	30.59 \pm 1.77	37.01 \pm 2.70	36.51 \pm 4.24	34.70 \pm 1.79	—	ns

B.

TNF- α (ng/mL)	EL ($n = 7$)	PL ($n = 7$)	ML ($n = 7$)	Average ($n = 21$)	II. Control vs. TNF- α
0	28.02 \pm 1.22	31.61 \pm 2.44	27.29 \pm 2.75	28.97 \pm 1.29	—
1	31.16 \pm 2.11	35.81 \pm 3.23	31.37 \pm 3.24	32.78 \pm 1.66	$P < 0.001$
10	33.29 \pm 2.22	36.94 \pm 3.08	33.40 \pm 3.45	34.54 \pm 1.67	$P < 0.001$
50	35.01 \pm 2.16	39.63 \pm 3.15	36.24 \pm 4.15	36.96 \pm 1.84	$P < 0.001$

ns — Not significant

Experiments were performed on 49 clinically healthy, Holstein-Friesian dairy cows, in their 1st to 4th lactation, from the Ghent University dairy herd (Biocentrum Agri-Vet, Melle, Belgium). Three groups of cows were sampled: 15 cows in EL (15.1 ± 1.95 d after parturition), 15 in PL (57.7 ± 3.62 d after parturition), and 19 in ML (133 ± 3.63 d after parturition). Immediately after morning milking, blood was aseptically collected in pyrogen-free heparinized vacuum tubes (Chromogenix, Milano, Italy) by jugular venipuncture between 08:00 and 09:00. Smears were prepared from whole blood and stained (Hemacolor; Merck, Darmstadt, Germany). Differential microscopic counts were determined by counting 200 cells. Only samples with PMN counts greater than 90% of the total granulocyte population were included in the study.

Recombinant human TNF- α (Calbiochem, San Diego, California, USA) was diluted in phosphate buffered saline solution (Dulbecco's PBS [DPBS]; Gibco, Paisley, United Kingdom) containing 0.1% fetal calf serum (Sigma, Bornem, Belgium) and stored in aliquots at -70°C until use. Anti-bovine TNF- α monoclonal antibody (mAb) (13) was diluted in DPBS. Lipopolysaccharides (*Escherichia coli* O111:B4; Sigma-Aldrich) were dissolved in 0.9% saline.

To test the effect of the different compounds, pyrogen-free polystyrene round-bottom tubes (Becton Dickinson, San José, California, USA) were used containing 90 μ L whole blood. Samples were pre-incubated with 10 μ L anti-bovine TNF- α mAb for 15 min at room temperature. After the addition of LPS (1 to 10⁴ ng/mL) or TNF- α (1 to 50 ng/mL), concentrations routinely used for in vitro experiments, all samples were incubated for 90 min at 37°C. Further incubation for 30 min at 37°C was done with either 50 μ L anti-bovine Mac-1 (clone CC126 mAb; ProBio, Margate, Kent, United Kingdom) or 10 μ L anti-ovine CD14 (clone VMP65; Serotec, Oxford, United Kingdom) at saturating concentrations diluted in control solution, RPMI 1640 (Gibco Brl, Scotland, United Kingdom), supplemented

with 1% bovine albumin fraction V (Merck) and 0.2% NaN₃. Following incubation, indirect immunofluorescence was performed as previously described (14).

Specimens were analyzed on a FACScan flow cytometer (Becton Dickinson Immunocytometry Systems). For each sample, 20 000 events were recorded in list mode and displayed on a logarithmic scale. Leukocyte populations were characterized by forward and side light scattering characteristics, and dot plots were gated for PMN and monocytes. The PMN population was additionally identified by CH138A mAb (VMRD, Pullman, Washington, USA), which recognizes bovine granulocytes. The mean fluorescence intensity (MFI) and percent of positive cells in these areas were calculated after plotting the fluorescein isothiocyanate (FITC) fluorescence histograms. Nonspecific background fluorescence was any fluorescence associated with leukocytes incubated with FITC-labeled secondary antibody alone.

Basal levels of CD11b and CD14 were compared for the 3 stages of lactation by analysis of variance. The MFI of CD11b was statistically processed as a function of the LPS and TNF- α concentrations using a mixed model. Cow was introduced as a random effect, while lactation stage, LPS and TNF- α concentrations, and their interactions were added as categorical fixed effects. Pairwise comparisons were adjusted by Tukey's method. This model was also fitted in the presence of anti-bovine TNF- α mAb.

CD11b density on unstimulated blood PMN was evaluated in 9 cows per stage of lactation. A slightly lower, but non-significantly altered, MFI was found during EL (28.4 ± 1.03) as compared to PL (32.6 ± 2.07) and ML (30.4 ± 2.88). A similar high proportion of circulating PMN with positive staining for CD11b ($\sim 97\%$) was detected during the 3 stages of lactation. Decreased CD14 surface density on monocytes ($P < 0.05$) was found in EL (9.22 ± 1.01) versus PL (14.68 ± 2.12) or ML (14.26 ± 1.38) on resting PMN as evaluated in

11. Burton JL, Kehrl ME Jr, Kapil S, Horst RL. Regulation of L-selectin and CD18 on bovine neutrophils by glucocorticoids: effects of cortisol and dexamethasone. *J Leukoc Biol* 1995;57:317-325.
12. Filep JG, Delalandre A, Payette Y, Földes-Filep E. Glucocorticoid receptor regulates expression of L-selectin and CD11/CD18 on human neutrophils. *Circulation* 1997;96:295-301.
13. Paape MJ, Rautiainen PM, Lilius EM, et al. Development of anti-bovine TNF- α mAb and ELISA for quantitating TFN- α in milk after intramammary injection of endotoxin. *J Dairy Sci* 2002;85:765-773.
14. Diez-Fraile A, Meyer E, Duchateau L, Burvenich C. L-selectin and β_2 -integrin expression on circulating bovine polymorphonuclear leukocytes during endotoxin mastitis. *J Dairy Sci* 2003;86:2334-2342.
15. Mehrzad J, Dosogne H, Meyer E, et al. Respiratory burst activity of blood and milk neutrophils in dairy cows during different stages of lactation. *J Dairy Res* 2001;68:399-415.
16. Kishimoto TK, Jutila MA, Berg EL, Butcher EC. Neutrophil Mac-1 and Mel-14 adhesion proteins are inversely regulated by chemotactic factors. *Science* 1989;245:1238-1241.

17. Werling D, Jungi TW. TOLL-like receptors linking innate and adaptative immune response. *Vet Immunol Immunopathol* 2003;91:1–12.
18. Roets E, Burvenich C, Diez-Fraile A, Noordhuizen-Stassen EN. Evaluation of the role of endotoxin and cortisol on modulation of CD18 adhesion receptors in cows with mastitis caused by *Escherichia coli*. *Am J Vet Res* 1999;60:534–540.
19. Amar S, Oyaisu L, Li L, Van Dyke T. Moesin: a potential LPS receptor on human monocytes. *J Endotoxin Res* 2001;7: 281–286.
20. Adams JL, Czaprynski CJ. Bacterial lipopolysaccharide induces release of tumor necrosis factor- α from bovine peripheral blood monocytes and alveolar macrophages in vitro. *J Leukoc Biol* 1990;48:549–556.
21. Sordillo LM, Pighetti GM, Davis MR. Enhanced production of bovine tumor necrosis factor- α during the periparturient period. *Vet Immunol Immunopathol* 1995;49:263–270.
22. Preisler MT, Weber PSD, Tempelman RJ, et al. Glucocorticoid receptor expression profiles in mononuclear leukocytes of periparturient Holstein cows. *J Dairy Sci* 2000;83:38–47.